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Functions of Basic Fibroblast Growth Factor and Neurotrophins in the Differentiation of Hippocampal Neurons

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Summary

Restrictions in neuronal fate occur during the transition from a multipotential to a postmitotic cell. This and later steps in neuronal differentiation are determined by extracellular signals. We report that basic fibroblast growth factor is mitogenic for stem cells and is a differentiation factor for calbindin-expressing hippocampal neurons. The neurotrophin NT-3 is a differentiation factor for the same neurons but does not affect proliferation. NT-3 and brain-derived neurotrophic factor promote the maturation of neurons derived from stem cells that have been grown in vitro. These results define functions for basic fibroblast growth factor and neurotrophins in the differentiation processes that direct a multipotential stem cell to a specific neuronal fate.

Introduction

During the development of the nervous system, precursors give rise to different types of neuronal and glial cells on a precise schedule (McKay, 1989; Anderson, 1993; Barres and Raff, 1994). Fate mapping experiments show that many neurons and glia in the CNS are derived from multipotential cells (Turner and Cepko, 1987; Gray et al., 1988; Wetts and Fraser, 1988). The multipotent nature of the precursor cells stresses the role of extracellular signals in regulating the switch from stem cell to differentiated product. Transplantation experiments in the cerebral cortex show that these signals act at the time a dividing cell differentiates to a postmitotic neuron (McConnell and Kaznowski, 1991). These results emphasize the need to define ligand-receptor systems that regulate early steps in neuronal differentiation.

Conditions that allow the proliferation and differentiation of neural precursors are necessary for the analysis of mechanisms and factors regulating cell differentiation and fate choice. In culture, precursor cells derived from the mammalian CNS can proliferate and, under some conditions, differentiate into neurons and glia (Raff et al., 1983; Temple, 1989; Cattaneo and McKay, 1990; Reynolds et al., 1992; Davis and Temple, 1994). Oligodendrocyte precursors derived from the rat optic nerve require a combination of signals to differentiate into postmitotic cells (for a review, see Barres and Raff, 1994). In the sympathoadrenal lineage, the continued action of different signals may synergistically promote neuronal differentiation, nerve growth factor (NGF) dependence for survival, and maturation of sympathetic neurons (for a review, see Anderson, 1993; Ip et al., 1994). The differentiation of neuronal cells in the CNS is not so well understood. The experiments reported here define the functions of soluble factors that regulate different steps in the transition from a precursor to a neuron in the hippocampus.

High levels of basic fibroblast growth factor (bFGF) and its receptors are found during mitotically active phases of chicken and rat nervous system development (González, et al., 1990; Wanaka et al., 1991; Peters et al., 1992). The expression of neurotrophin-3 (NT-3), brain-derived neurotrophic factor (BDNF; Maisonpierre et al., 1990; Ernfors et al., 1992), and tyrosine receptor kinase (Trk) receptors (Klein et al., 1990; Tessarollo et al., 1993) in the hippocampal formation suggests that these neurotrophins act on postmitotic neurons rather than in proliferative cells. bFGF stimulates the proliferation and survival of hippocampal cells (Walicke et al., 1986; Collazo et al., 1992; Ohsawa et al., 1993; Ray et al., 1993). NT-3 is a neurotrophic factor for calbindin-positive hippocampal neurons (Collazo et al., 1992; Ip et al., 1993). Though these reports show that bFGF and NT-3 are neurotrophic for hippocampal neurons, they do not distinguish among differentiation, maturation, and survival mechanisms of action. In this paper, we investigate more precisely the cellular targets and functions of neurotrophins and bFGF in the development of hippocampal neurons.

Hippocampal cells were grown under two conditions: acutely dissociated from rat embryonic day 16 (E16) embryos and after in vitro passaging of the cells in the presence of bFGF. Cells were analyzed for the expression of stem cell, neuronal, and glial markers at different days in culture. The ability of acutely dissociated and expanded precursor cells to differentiate in response to bFGF and neurotrophins was assessed. Our results show that both bFGF and NT-3 are differentiation factors for calbindin-expressing hippocampal neurons. bFGF is also a mitogen, stimulating the proliferation of a cell that expresses nestin, a marker of neuroepithelial stem cells (Hockfield and McKay, 1985; Frederiksen and McKay, 1988; Cattaneo and McKay, 1990; Lendahl et al., 1990; Zimmerman et al., 1994). Nestin-positive cells that have been expanded in vitro can differentiate to express neuronal antigens and morphologies in response to neurotrophins. These results suggest that bFGF, NT-3, and BDNF interact to promote the differentiation of hippocampal neurons from their proliferating stem cells.

Results

Effects of bFGF and NT-3 on Hippocampal Cells

Effect of bFGF on Cell Number and Type

The effect of increasing concentrations of bFGF on the labeling index (bromodeoxyuridine [BrdU] incorporation) was tested in short-term cultures before the cells were passaged and in cells that were passaged twice. Figure 1a shows that in both cases, increasing concentrations of bFGF from 0 to 5 ng/ml produced a 2- to 3-fold increase

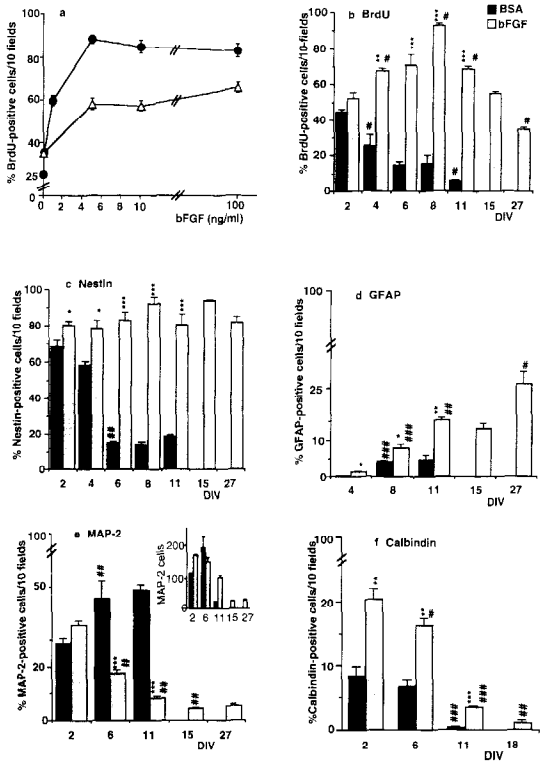

Figure 1. Characterization of the Effects of bFGF on Hippocampal Primary Cells

(a) Dose–response curve of the bFGF effect on the BrdU incorporation of primary hippocampal cells in culture. Cells were prepared from rat embryonic hippocampus on E16 and plated onto polyornithine-coated well chambers at 45,000 cells/cm^2. Cells were grown in N2 medium and varying concentrations of bFGF during 5 days without passage (passage 0; circles). For passage 2 conditions (triangles), cells were passaged twice in 11 days in bFGF at 5 ng/ml and then incubated with varying concentrations of bFGF for another 5 days. bFGF was replaced every 2–3 days. BrdU was added to the cultures at a final concentration of 10 μM for 18 hr prior to fixation. Cells were immunostained with anti-BrdU antibody.

(b–e) Identification of hippocampal cells growing in bFGF. Cells were grown in N2 medium in the presence of 5 ng/ml bFGF or BSA. At different days in vitro (DIV), cells were fixed and immunostained for BrdU (b), nestin (c), GFAP (d), MAP2 (e), or calbindin (f). In all panels, a total of 10 fields were counted under a phase-contrast microscope using 400× magnification. Results are expressed as percentage of cells expressing a specific marker per 10 fields or, in inset of (e), as the number of cells expressing MAP2.

For each condition, 4–5 samples were tested, and the data are the average ± SEM of 2 experiments. During the entire period of study, 80%–90% of the cells maintained in bFGF expressed nestin (c), an intermediate protein characteristic of neuroepithelial cells. Single asterisk, $p < .05$; two asterisks, $p < .01$; three asterisks, $p < .001$ (statistical significance of bFGF data versus BSA controls). Single pound sign, $p < .05$; two pound signs, $p < .01$; three pound signs, $p < .001$ (statistical significance of each value versus previous value). Shills indicate broken scale.

in the percentage of BrdU-positive cells in the cultures. Much higher concentrations of bFGF (100 ng/ml) showed no significant increase in the percentage of BrdU-positive cells. Therefore, a saturating dose of 5 ng/ml bFGF was used throughout this study.

To test whether the effect of bFGF on the proliferative capacity of these cells changed with time, the labeling

Table 1. Antigen Expression and BrdU Labeling by Acutely Dissociated Hippocampal Cells Growing in bFGF

Markers	Percentage of Labeled Cells
Nestin$^+$/BrdU$^+$	91
Calbindin$^+$/Nestin$^+$	25
Calbindin$^+$/BrdU$^-$	94

Cells were prepared from rat embryonic hippocampus on E16 and plated onto polyornithine chambers at 45,000 cells/cm^2. Cells were grown in N2 medium in the presence of 5 ng/ml bFGF for 3 days and exposed to 10 μM BrdU during 18 hr prior to fixation. Cells were fixed and double-stained with anti-nestin, anti-BrdU, or anti-calbindin antibodies and visualized by using rhodamine- and fluorescein-conjugated secondary antibodies. A total of 10 fields were counting under a fluorescence microscope using 200 × magnification. The values are percentage of nestin cells that are BrdU positive or percentage of calbindin cells that are nestin positive or BrdU negative.

index was measured in the presence and absence bFGF cells at various times in culture. Figure 1b shows that after 2 days in vitro (DIV), the proportion of BrdU-positive cells was similar (about 50%) in cells grown with and without bFGF. In the first week in culture, the percentage of cells that incorporated BrdU in the presence of bFGF increased from 50% to 90%. After 2 weeks in culture, there was a decrease in the proliferative capacity of the bFGF-expanded cells, declining to a labeling index of 30% at the last timepoint examined (27 days). In the absence of added bFGF, there is a rapid decrease in labeling index, and cells die. These results are consistent with a a simple model in which high affinity receptors for bFGF are directly responsible for proliferation.

To determine the phenotype of the cells that proliferate and survive in the presence of bFGF, a panel of antibodies was used. Antibody against nestin, an intermediate filament protein, was used to recognize neural precursor cells (Hockfield and McKay, 1985; Frederiksen and McKay, 1988; Lendahl et al., 1990; Tohyama et al., 1992; Zimmerman et al., 1994). Neurons were identified by the expression of microtubule-associated protein 2 (MAP2; Matus et al., 1986). In the hippocampus, many dentate granule neurons and some pyramidal cells and interneurons express the calcium-binding protein calbindin (Sloviter, 1989). Therefore, an antibody against this protein was used to detect this neuronal subtype. Antibody against glial-fibrillary acidic protein (GFAP) was used as a general marker for astrocytes (Bignami et al., 1972).

Figure 1c shows that in the presence of bFGF, there is a persistent expression of nestin, ranging from 80%–90% of the cells during the entire time course examined. In cultures examined 3 days after plating, more than 90% of the nestin-positive cells were also BrdU positive (Table 1). After 6 DIV, most of the nestin-expressing cells growing in bFGF showed small pyramidal morphology bearing short processes (unpublished data). Nestin expression decreased in the bovine serum albumin controls, and after 6 days, only about 15% of the cells were nestin positive (Figure 1c). The correlation between nestin expression and proliferation suggests that cells growing in the ab-

sence of bFGF down-regulate nestin and stop proliferating.

The fraction of cells expressing GFAP increased during prolonged exposure of hippocampal cultures to bFGF (Figure 1d). Initial GFAP expression in these cultures is low (1%). The percentage of GFAP-expressing cells increased in cultures treated with bFGF compared with controls, reaching 25% after 27 DIV. In this culture system, GFAP-positive/nestin-positive cells, as well as GFAP-positive/nestin-negative cells, were present (data not shown). These results show that bFGF promotes the proliferation of neuroepithelial cells that give rise to astrocytes.

In the presence of bFGF, there was a significant decrease over time in the percentage of cells expressing the neuronal antigen MAP2 (Figure 1e). After the second week in culture, about 5% of the cells were MAP2 positive. This observation was reproduced using other neuronal antigens, such as neuron-specific enolase and neurofilament (data not shown). During the first 6 DIV, the decrease in the proportion of MAP2-positive cells can be explained by the increase of cells expressing nestin (Figure 1c). When hippocampal cells were grown in the presence of bFGF for 2 weeks without passaging, approximately 85% of the cells were MAP2 negative (data not shown). This result indicates that the increase in nestin-positive cells is not a consequence of passaging the cultures.

In the control cultures, the proportion of cells expressing MAP2 increased over time, reaching 50% at 6 days in culture. There is a reciprocal loss of nestin-positive cells. These data show that neurons differentiate when dissociated cells are placed in culture. bFGF does not influence this process, as there is little effect on the number of MAP2-positive cells. At 11 days, most of the cells die in the absence of bFGF (Figure 1e, inset). These results show that bFGF does not increase the total number of neurons, but it is a survival factor for hippocampal neurons, as has previously been reported (Walicke et al., 1986) and can be seen at 11 DIV in Figure 1e (inset).

In contrast, bFGF has specific neurotrophic effects on a subset of hippocampal cells, the calbindin-expressing neurons (Collazo et al., 1992; Ip et al., 1993). A time course of the effects of bFGF on calbindin-positive neurons shows that the fraction of cells expressing calbindin was 2.5- to 8-fold greater in the bFGF cultures than in the controls (Figure 1f). During the first week, 15%–20% of the cells were calbindin positive. bFGF increases the number of calbindin-expressing cells by 2 days in culture. However, the effect of bFGF on MAP2-positive cells was first visible after a week in culture (Figure 1e, inset). Experiments described below extend these observations and show that bFGF acts as a differentiation factor on calbindin-positive neurons.

Effect of bFGF and NT-3 on Calbindin Expression by Hippocampal Neurons

The trophic effects of bFGF and NT-3 on hippocampal calbindin-expressing neurons raise three further questions. First, are these neurotrophic effects a consequence of a differentiation pathway triggered by the growth factor or a survival effect on a differentiation program that is initiated by other signals? Second, do NT-3 and bFGF act on the same population of responding cells? Third, if the same cells respond to the factors, do independent receptor systems mediate the neurotrophic effects of bFGF and NT-3?

The short-term effects of bFGF on calbindin-positive cells over the first 6 DIV (Figure 1f) could be explained by

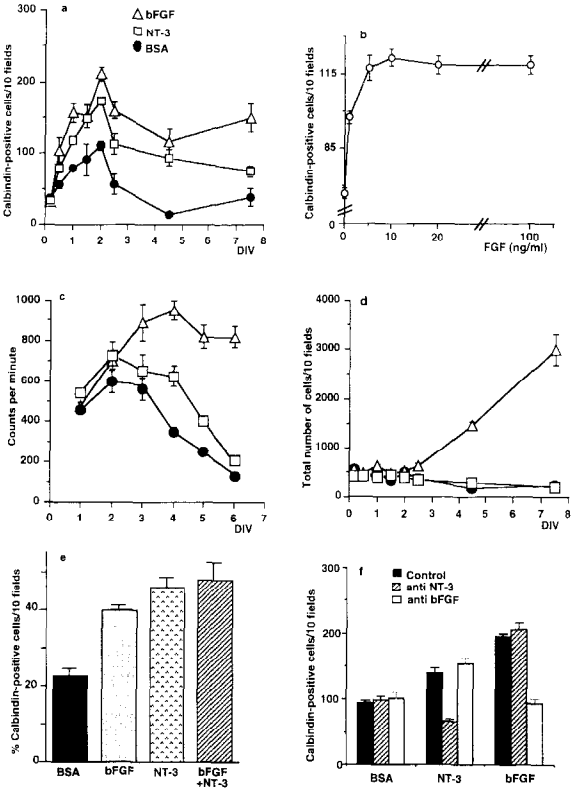

Figure 2. Induction of Calbindin Cell Differentiation Promoted by bFGF and NT-3

(a) rapid induction of calbindin expression by bFGF and NT-3 in short-term cultures. Addition of growth factors produced a 5- to 7-fold increase in the number of calbindin-expressing cells during the first 2 days.

(b) The induction of calbindin by bFGF was saturated at concentrations equal to or greater than 5 ng/ml.

(c and d) During the first 2 DIV, no differences among bFGF, NT-3, or controls on [^{3}H]thymidine incorporation by the cells (c) or in the total number of cells (d) were observed.

(e) The percentage of calbindin-positive cells at the time when maximal levels were found, 2 days. bFGF plus NT-3 produced the same increase in calbindin-positive cells as either factor added alone.

(f) Selective blocking of calbindin induction by specific antibodies against bFGF or NT-3.

Hippocampal cells were prepared and plated as described in Figure 1. Cells were grown in N2 medium in the presence of varying concentrations of bFGF, 20 ng/ml NT-3, 5 ng/ml bFGF + 20 ng/ml NT-3, or BSA for the indicated number of days. Anti-bFGF (1:100) and anti-NT-3 (1:100) antibodies were added to the cells at the time of plating and replaced every day for 2 days. [^{3}H]thymidine was added to the cells at a final activity of 2 μCi/ml. At different DIV, cells were fixed and immunostained for calbindin-D_{28kDa}. A total of 10 fields were counted under a phase-contrast microscope using 200× magnification. Results are expressed as number or percentage of cells expressing calbindin, as the total number of cells per 10 fields, or as incorporated counts per minute. For each condition, 4–5 samples were tested, and the data are the average ± SEM of 2 experiments. Shills indicate broken scale.

either a survival or differentiation action. The effect of bFGF and NT-3 on calbindin expression at short times in culture was measured to establish whether these factors act as differentiation agents. As shown in Figure 2a, both bFGF and NT-3 rapidly increased the number of calbindin-expressing cells (7-fold and 5-fold, respectively), reaching maximum levels after 2 days in culture. In bovine serum albumin (BSA) controls, there was a 3-fold increase during the same period of time.

There is strong evidence that the differentiation and proliferative functions of bFGF are independent. The dose–response curves for the proliferative and differentiation effects of bFGF are similar (Figure 1a and Figure 2b), suggesting that both functions are directly mediated by high affinity receptors. When E16 hippocampal cells are analyzed immediately after dissection, more than 80% of the cells are nestin positive. Though this level of nestin expression is maintained by bFGF, only 25% of the calbindin-positive cells express nestin after 3 days in culture (Table 1), suggesting that there are two populations of cells that respond to bFGF, either by proliferating or by differentiating.

This conclusion is supported by additional data. Over 90% of the calbindin-positive cells are not proliferating in these short-term cultures, as measured by BrdU incorporation (Table 1). During the first 2 days in culture, bFGF and NT-3 have no effect on the thymidine incorporation, nor do they influence the number of cells (Figures 2c and 2d). These data are consistent with a model in which tyrosine kinase receptors for bFGF and NT-3 can stimulate calbindin expression in the early stages of neuronal differentiation.

When bFGF and NT-3 were added together to cultured cells, the response was not additive (Figure 2e). The lack of an additive effect shows that the same population of cells responds to bFGF and NT-3. To test whether bFGF and NT-3 induced calbindin expression by independent pathways, we used specific antibodies that block the function of NT-3 and bFGF. Blocking antibodies against NT-3 or bFGF inhibited the increase in the number of calbindin-expressing cells without affecting the response to the other growth factor (Figure 2f). Antibodies did not have significant effect on the total number of cells (data not shown). These results suggest that the NT-3 and bFGF effects are mediated by independent receptors on the same cells.

These results show that NT-3 and bFGF cause an increase in the number of calbindin-positive cells within hours after plating, and this effect is maintained for several days (Figure 2a). The longer term effects of these extracellular signals show that there is a survival effect on calbindin-positive neurons. Double-labeling studies show that calbindin-positive neurons up-regulate c-Fos in response to NT-3 (Collazo et al., 1992), suggesting that the survival effect is directly mediated by Trk receptors on the neurons. Similar studies are necessary to determine whether the survival effects of bFGF are mediated by receptors on the neurons or indirectly through another cell.

Differentiation of Cells Expanded in bFGF

The results above show that bFGF and NT3 are differentiation factors in acutely dissociated hippocampal cultures,

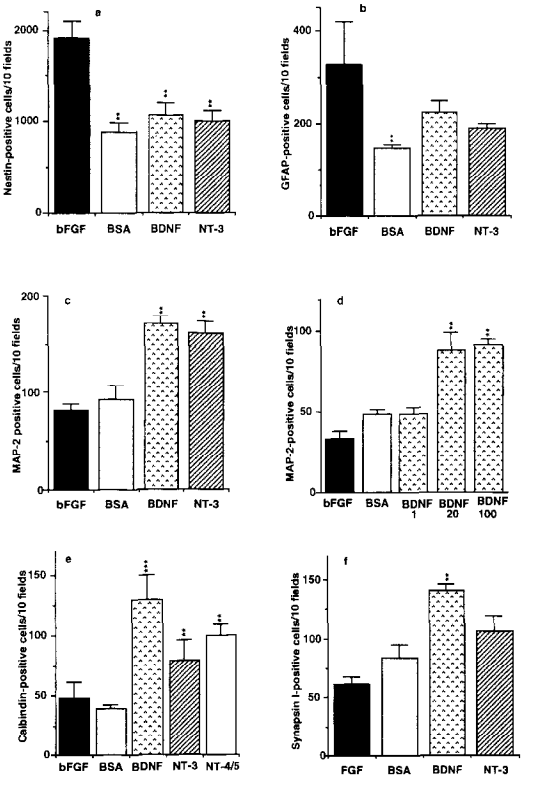

Figure 3. BDNF and NT-3 Increase the Expression of Neuronal Markers on Hippocampal Cells Expanded in bFGF

Hippocampal cells were prepared and plated as described in Figure 1. Cells were passaged twice in 13 days in N2 medium in the presence of 5 ng/ml bFGF. Then, bFGF was removed, and cells were washed and incubated for an additional 5 days in the presence of 20 ng/ml BDNF, 20 ng/ml NT-3, or 20 ng/ml NT-4/5. As controls, some cells were maintained in bFGF or switched to BSA for 5 days. In (d), concentrations of BDNF of 1 ng/ml and 100 ng/ml were also tested after the third passage of the cells. Growth factors were replaced every 2 days. Cells were fixed and immunostained for nestin (a), GFAP (b), MAP2 (c and d), calbindin-D_{28kDa} (e), or synapsin I (f). A total of 10 fields were counted under a phase-contrast microscope using 200× magnification (nestin, GFAP, MAP2) or 100× magnification (calbindin and synapsin I). Lower magnification in the counting of calbindin-positive or synapsin I–positive cells was chosen to increase the level of confidence. In the presence of BDNF, the MAP2-positive cells and calbindin-positive cells represent 12% and 5%, respectively, of total cells. Results are expressed as the number of cells expressing a specific marker per 10 fields. For each condition, 4–5 samples were tested, and the data are the average ± SEM of 2 experiments. Single asterisk, $p < .05$; two asterisks, $p < .01$; three asterisks, $p < .001$ (statistical significance of each data versus BSA values).

and that the same cells can respond to either factor by expressing calbindin. These results contrast with the proliferative effects of bFGF, as most of the cells growing in bFGF remained as neural precursor cells according to the expression of nestin and lack of expression of neuronal antigens. The simplest interpretation of this data is that acutely dissociated hippocampal cultures contain two bFGF-responsive cells: a cell that proliferates and a cell that differentiates. The differentiation functions of NT-3 on acutely dissociated cells, along with the neurotrophic roles of NT-3, BDNF, and NT-4/5 on neuronal cells (Alderson et al., 1990; Collazo et al., 1992; Segal et al., 1992; Ip et

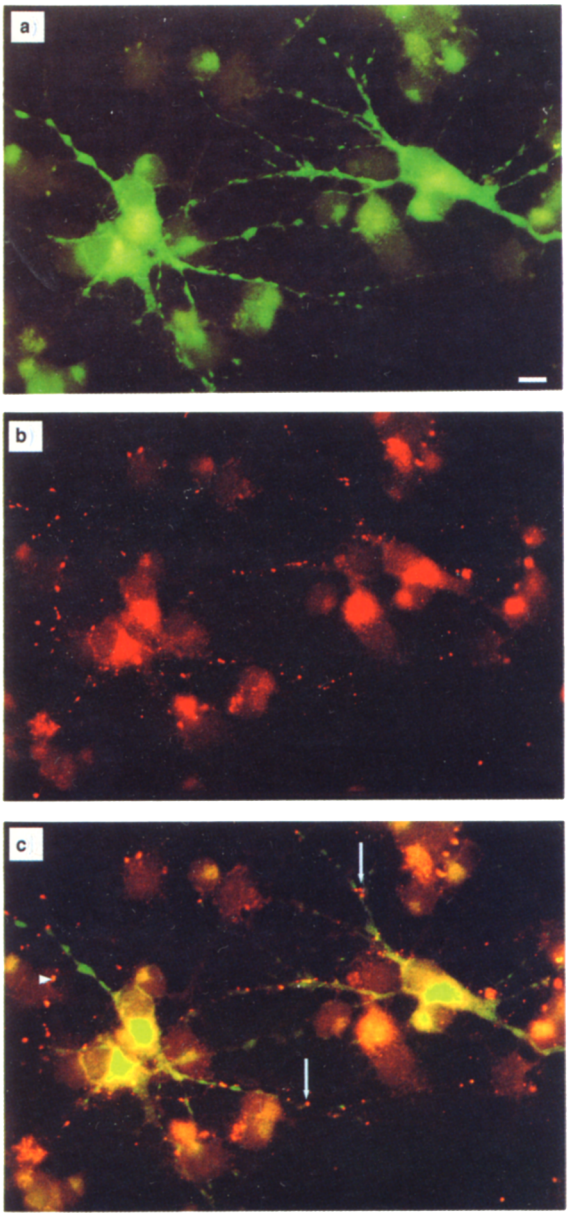

Figure 4. BDNF Increases the Number of Synapsin I–Positive Neurons on the bFGF-Expanded Cells

Hippocampal cells were prepared and plated as described in Figure 3. Cells were fixed and immunostained for MAP2 and synapsin I.
(a) MAP2-positive cells visualized using fluorescein-conjugated secondary antibody present complex morphologies, having pyramidal cell bodies and bearing long processes.
(b) Synapsin I accumulates in puncta in close contact with processes as visualized using rhodamine-conjugated secondary antibody.
(c) Double exposure for the MAP2 and synapsin I staining reveals that most of the synapsin-rich puncta do not show MAP2 staining as indicated by the orange-red color (arrows) instead of the yellow (arrowhead).
Bar, 10 μm.

al., 1993; Ghosh et al., 1994), prompted us to test their effects on the bFGF-expanded cells.

Cells growing in bFGF for 13–18 days (passages 2 and 3) were switched to BDNF, NT-3, NT-4/5 (20 ng/ml), BSA, or maintained in bFGF for another 5 days. The removal of the mitogen produced a major decrease in the number of nestin-positive cells (Figure 3a). The number of cells expressing the astrocytic marker GFAP was also reduced by 30%–40% in neurotrophin conditions and approximately 55% in BSA (Figure 3b). These results show that removal of the mitogen reduces the number of cells expressing nestin or GFAP and that neurotrophins have little effect in these assays.

Because the loss of nestin expression in vivo and in vitro correlates with the transition of neuronal stem cells to neurons (Frederiksen et al., 1988; Frederiksen and McKay, 1988; Cattaneo and McKay, 1990; Hayes et al., 1991), it is interesting to ask whether the in vitro expanded nestin-positive cells can also acquire neuronal properties. Cells were analyzed for expression of MAP2, calbindin, and synapsin proteins and for morphological changes in response to bFGF withdrawal and neurotrophin addition. Simply withdrawing bFGF has little effect on the number of MAP2-positive cells (Figures 3c and 3d), and calbindin-expressing cells (Figure 3e). In contrast, expression of MAP2 was enhanced by 2-fold in the presence of BDNF or NT-3, reaching saturation at 20 ng/ml of neurotrophic factor (Figure 3d). BDNF, NT-3, and NT-4/5 also increased the number of calbindin-positive cells by 3-fold, 2-fold, and 2.5-fold, respectively, compared with BSA (Figure 3e). In the presence of BDNF, 12% of the cells expressed neuronal markers.

Changes in the expression of synaptic proteins and neuronal morphology during development have been related to different stages of neuronal maturation (Dotti et al., 1988; Fletcher et al., 1991; Verdi et al., 1994). Removal of bFGF and addition of BDNF leads to a 70% increase in the number of cells expressing synapsin I compared with BSA (Figure 3f). Synapsin is mostly concentrated in puncta, often associated with MAP2-positive processes, but is not colocalized with that protein (Figure 4). Note that the effect of BDNF is more pronounced than the effect of NT-3 in this assay. Neurotrophin-treated cells showed a significantly larger cell body area, a greater number of processes and branches, and longer processes compared with cells switched to BSA or maintained in bFGF, as shown in Table 2 and Figure 5. These results show that cells expanded in bFGF express antigens and morphologies characteristic of differentiating hippocampal neurons in response to NT-3 and BDNF.

Discussion

Experiments with single cell cultures of primary cells and clonal cell lines show differentiation to both neuronal and glial fates in vitro and in vivo (Temple, 1989; Frederiksen et al., 1988; Renfranz et al., 1991; Snyder et al., 1992; Davis and Temple, 1994). These experiments emphasize the multipotential nature of neuroepithelial cells in the CNS. They raise interesting questions about the signals that regulate the transition from proliferating cell to a postmitotic, differentiated product. The experiments reported here analyze the proliferation and differentiation of hippocampal cells under two conditions: acutely dissociated from E16 embryos and after in vitro expansion in the presence of bFGF. Growth factors that act on these cells as

Table 2. BDNF and NT-3 Promoted Neuronal Maturation of bFGF-Expanded Calbindin Cells

Condition	Cell Body Area (μm^2)	Number of Processes and Branches	Length of Longest Process (μm)
bFGF	74.3 ± 3.7 (110)	6.2 ± 0.5 (117)	98.4 ± 13.3 (144)[a]
BSA	67.3 ± 4.0 (100)	5.3 ± 0.5 (100)	68.0 ± 6.4 (100)
BDNF	91.3 ± 3.4 (136)[b]	8.4 ± 0.4 (159)[b]	138.3 ± 13.2 (203)[b]
NT-3	95.9 ± 4.3 (142)[b]	9.0 ± 0.5 (170)[b]	124.7 ± 11.1 (183)[b]

E16 hippocampal cells were grown and immunostained for calbindin-D_{28kDa}, as described in Figure 3. Cells were examined on an inverted microscope set up for differential interference contrast optics with a 40× objective using bright-field optics. The images were collected with a videocamera and processed to enhance contrast using a computer-based system. The cell body area, number of processes and branches, and length of longest process of 30 cells for each condition were measured. Results are expressed as average ± SEM. Given in parentheses is the percentage for each value versus BSA conditions (100%).

[a] $p < .01$.

[b] $p < .001$ (statistical significances versus BSA values).

mitogens and differentiation agents are defined. bFGF causes the proliferation of neuroepithelial cells expressing the intermediate filament nestin. bFGF and NT-3 induce neuronal differentiation by acting on early postmitotic cells. NT-3 and, more effectively, BDNF promote maturation of cells that have been expanded in vitro to express neuronal morphologies and synaptic antigens. These experiments identify functions for extracellular signals in the mechanisms that control differentiation of a specific subset of hippocampal neurons from proliferating precursors.

bFGF Stimulates Proliferation of Nestin-Positive Cells

bFGF has been shown to have trophic and proliferative effects on hippocampal primary cells, although its cellular targets have not been precisely defined (Walicke et al.,

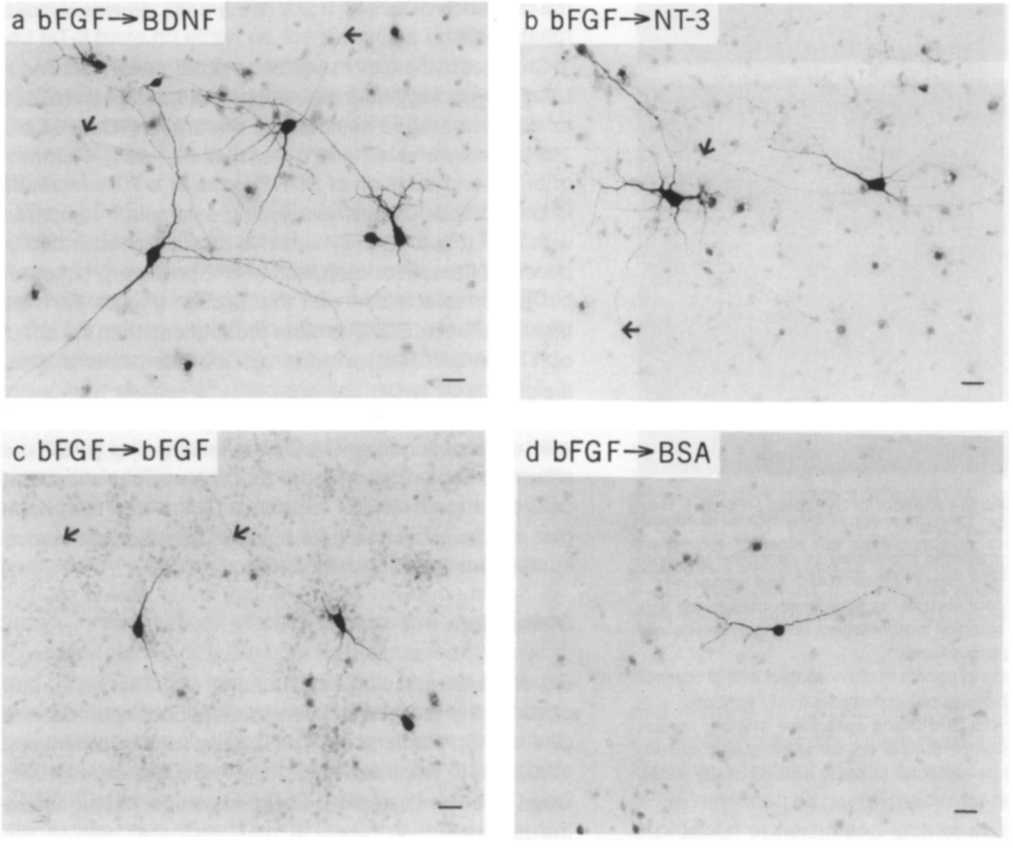

Figure 5. Calbindin-Expressing Cells Growing in the Presence of bFGF Show a More Mature Morphology when Switched to Neurotrophins

In the absence of bFGF, the addition of BDNF (a) or NT-3 (b) to the cultures promoted the acquisition of a more elaborate neuronal morphology by the calbindin-expressing cells as compared with cells maintained in bFGF (c) or switched to BSA (d). See Table 2 for measurements of the cell body area, number of processes and branches, and length of the longest process. Arrows indicate long processes for which the antibody staining was weak. Hippocampal cells were prepared and plated as described in Figure 3. Cells were fixed and immunostained for calbindin-D_{28kDa} and visualized by using avidin-biotin-horseradish peroxidase complex and DAB under bright-field optics using 200× magnification. Bar, 25 μm.

1986; Collazo et al., 1992; Ohsawa et al., 1993; Ray et al., 1993). bFGF exerted a strong proliferative effect on hippocampal cells, so that after a month in culture, 30% of the cells incorporated BrdU (see Figure 1b). Even though bFGF was able to maintain cell proliferation, there was a progressive decrease in the labeling index after the first 2 weeks in vitro, which was not prevented by addition of high concentrations of the growth factor, such as 100 ng/ml (see Figure 1a). Astrocytic differentiation occurring in the culture may alter the proliferative responses to bFGF (Gao et al., 1991).

One of the more striking features of the cells growing in bFGF is the large proportion of dividing cells expressing nestin (see Figures 1b and 1c; Table 1). These data and the decrease in the fraction of cells expressing neuronal markers (see Figure 1e) suggest that the hippocampal cell population that proliferates in response to bFGF is mainly composed of neuroepithelial stem cells. These and previous in vitro results (Deloulme et al., 1991; Ohsawa et al., 1993; Ray and Gage, 1994), along with the abundant expression of bFGF (González et al., 1990) and bFGF receptors (Wanaka et al., 1991; Peters et al., 1992) during phases of active cell proliferation in the CNS, suggest a developmental role for bFGF in stimulating the proliferation of neuroepithelial cells giving rise to neuronal and glial cells.

Consistent with this and in agreement with previous reports (Perraud et al., 1988; Ray and Gage, 1994), our results suggest that bFGF maybe a mitogen and/or differentiation factor for astrocyte precursors (see Figure 1d). The survival of GFAP-positive cells requires the continued presence of FGF, since withdrawal of the growth factor from the cultures was followed by astrocytic death (see Figure 3b). After 2 weeks in vitro, GFAP-positive cells can be distinguished by the presence or absence of nestin expression, suggesting that these cells represent two different stages in astrocytic differentiation.

bFGF and NT-3 Induce Calbindin Neuronal Differentiation

bFGF and NT-3 are survival factors for hippocampal calbindin-expressing neurons in vitro (Collazo et al., 1992; Ip et al., 1993). We have previously shown using a Fos-induction assay that calbindin-positive neurons respond directly to NT-3 (Collazo et al., 1992). The data reported here extend our understanding of the cellular targets for both bFGF and NT-3. After 3 days in culture, the cells that express calbindin are not proliferating, and nestin is down-regulated in the calbindin-expressing cell population (Table 1). The rapid induction of calbindin expression by bFGF and NT-3 (see Figure 2a) contrasts with the lack of effect on total cell number (see Figure 2d). These results show that bFGF and NT-3 act at early stages of neuronal differentiation and induce signaling pathways that specify a particular neuronal subtype. As suggested by the lack of additive effect (see Figure 2e), bFGF and NT-3 induce calbindin differentiation acting on the same cell population. Our results with blocking antibodies suggest that bFGF and NT-3 promote calbindin expression independently.

It is remarkable that in these hippocampal cultures, bFGF acts both as a mitogen and as a differentiation factor. It is likely acting on different cell populations, inducing proliferation in the nestin-expressing neuroepithelial cells and differentiation in the precursors for calbindin-positive neurons. The dose–response curves for BrdU incorporation and calbindin induction suggest that bFGF interacts with high affinity receptors to trigger diverse actions. It is important to establish how this switch in signaling is achieved.

Though there are many studies reporting the neurotrophic effects of neurotrophins (Korsching, 1993), our results show that NT-3 is a differentiation factor for calbindin-positive hippocampal neurons. Our data also suggest that after 2 days in vitro, both bFGF and NT-3 support the survival of calbindin-positive neurons. But it is the earlier effects on the differentiation of calbindin-positive neurons that are most thoroughly documented in the data presented here. The transition from proliferating stem cell to postmitotic neuron induces a signaling mechanism that links tyrosine kinase receptors to the differentiation of a specific neuronal subtype. Receptor number may play an important role, as recent studies show that elevated levels of EGF and insulin receptors can stimulate neuronal differentiation in PC12 cells (Dikic et al., 1994; Traverse et al., 1994). However, the data presented here provide some of the first evidence for the specific action of extracellular ligands during the transition from multipotential cell to differentiated neuron.

Mice lacking NT-3, BDNF, TrkC, or TrkB do not show gross abnormalities in the CNS (for a review see Klein, 1994). In the hippocampus, the expression of calbindin is decreased only in a small proportion of interneurons in mice lacking BDNF (Jones et al., 1994). An explanation for these results may be that the absence of one factor can be complemented by the presence of another factor. We report that bFGF and NT-3 have indistinguishable effects on the differentiation of calbindin-positive neurons.

Neuronal Maturation of Cells Expanded In Vitro

In vivo, nestin is expressed in mitotic neural precursor cells and is lost as postmitotic cells differentiate to neurons (Frederiksen and McKay, 1988; Hockfield and McKay, 1985; Zimmerman et al., 1994). In embryonic rat cortex, a parallel decline in the number of nestin-positive cells and in CDC2 mRNA coincides with neuronal differentiation (Hayes et al., 1991). In vitro data also show a reciprocal down-regulation of nestin expression and the acquisition of neuronal antigens and morphology (Cattaneo and McKay, 1990). Similarly, neuronal differentiation in acutely dissociated cells occurs with a reciprocal decrease in nestin expression (Table 1; see Figure 2a). Expanded cells in bFGF show high levels of nestin and low levels of neuronal markers, suggesting that most of the cells remain as precursors. A fraction of these cells differentiate to express glial markers (see Figure 1d). It is important to define quantitative assays that measure neuronal maturation in cells that have been expanded in vitro.

In cultures of bFGF-expanded hippocampal cells, withdrawal of the mitogen results in a marked decrease in

the number of nestin-positive cells (see Figure 3a). The addition of BDNF or NT-3 leads to a significant increase in the number of cells expressing MAP2 and calbindin (see Figures 3c, 3d, and 3e). The increase in the number of calbindin-positive neurons was also observed in the presence of NT-4/5 (see Figure 3e). Calbindin-positive cells exposed to neurotrophins showed larger cell bodies and a greater number of more elaborate processes than cells growing in bFGF or switched to BSA (Table 2; see Figure 5). Morphological changes can be related to different stages of neuronal maturation (Dotti et al., 1988; Verdi et al., 1994). Our results show that NT-3, NT-4/5, and BDNF are able to induce neuronal differentiation of the nestin-positive cells and/or promote the maturation of calbindin-positive cells expanded in bFGF. The pattern of synapsin I expression supports this conclusion. The role of neurotrophins promoting the maturation of hippocampal neurons parallels the reported actions of these factors on developing sensory neurons (Wright et al., 1992).

Sequential roles for neurotrophins in the differentiation, survival, and maturation of neuronal cells have been reported previously (Segal et al., 1992; Buchman and Davies, 1993; De la Rosa et al., 1994; Ip et al., 1994). Although BDNF has survival effects on acutely dissociated hippocampal calbindin-positive cells, its effect is lower and delayed compared with NT-3 (Collazo et al., 1992; Ip et al., 1993). Our results and previously published data (Ip et al., 1993) show that calbindin-positive cells are more responsive to BDNF with time in culture. This suggests that the main role of BDNF on development of these cells may not be during early stages of neuronal differentiation but at a later stage in neuronal maturation. Consistent with this view, BDNF is more effective than NT-3 in increasing the number of cells expressing synapsin I (see Figure 3f). Synapsin accumulates in puncta, often along and in close contact with MAP2 processes (see Figure 4), as has been previously described for neurons during maturation (Fletcher et al., 1991). The delayed effects of BDNF relative to NT-3 in these cultures are consistent with a delay in the expression of BDNF and *trkB* relative to NT-3 and *trkC* in the hippocampal formation (Ernfors et al., 1992; Klein et al., 1990; Maisonpierre et al., 1990; Tessarollo et al., 1993). Data from the developing cerebellum also show sequential roles for BDNF and NT-3, but in contrast to the hippocampus, BDNF acts before NT-3 in the differentiation of cerebellar granule neurons (Segal et al., 1992).

The data presented in this work show that cell types, stages in cell differentiation, and ligand responses can be defined in hippocampal cultures. In combination with genetic tools, this experimental system is suitable to characterize further the many steps that generate the ordered structure of the hippocampus from stem cells.

Experimental Procedures

Materials

The materials were purchased from the following sources: calcium and magnesium-free Hanks' balanced salt solution (CMF-HBSS), Dulbecco's modified Eagle's Medium (DMEM), DMEM/Nutrient mixture F-12 (DMEM/F12), penicillin-streptomycin, and trypsin from Gibco/Bio-Rad Laboratories (Grand Island, NY); trypsin and DNaseI (for preparing cell suspensions) from Worthington (Freehold, NJ); poly-L-ornithine, fetal bovine serum (FBS), horse and goat serum, insuline, apo-transferrin, putrescine, progesterone, sodium selenite, BSA, trypan blue, paraformaldehyde, 3,3′ diaminobenzidine (DAB), and monoclonal antibody anti-calbindin-D_{28kDa} from Sigma (St. Louis, MO). [^{3}H]thymidine was from New England Nuclear (Boston, MA). Polyclonal antibodies anti-calbindin and anti-synapsin I were gifts of Dr. P. C. Emson (Cambridge, UK) and Dr. M. B. Kennedy (Pasadena, CA). Monoclonal antibodies anti-MAP2 (a, b, and c forms) were a gift of Dr. S. Okabe (National Institutes of Health, Bethesda, MD) and Dr. N. Hirokawa (Tokyo, Japan) or purchased from Sigma. Monoclonal antibody anti-GFAP was from ICN Biomedicals (Costa Mesa, CA); monoclonal and polyclonal anti-nestin protein were provided by Dr. M. Marvin (National Institutes of Health, Bethesda, MD); BrdU was from Boehringer Mannheim (Mannheim, Germany); monoclonal antibody against BrdU was from Becton Dickinson, (San Jose, CA); Vectastain ABC kit was from Vector laboratories (Burlingame, CA); goat antimouse IgG horseradish peroxidase conjugate was from Bio-Rad (Richmond, CA); fluorescence secondary antibodies were from Jackson Immuno Research (West Grove, PA) and Cappel (Durham, NC); Immu-mount was from Shandon (Pittsburgh, PA); DABCO was from Aldrich (Milwaukee, WI).

Hippocampal Cell Culture

The hippocampi from fetal brains of Sprague-Dawley rats at E16 (Taconic, Germantown, NY) were dissected free from the choroid plexus and meningeal tissue (Collazo et al., 1992). The hippocampi were then placed in ice-cold CMF-HBSS containing 0.01% DNAseI (1700–3300 U/mg) and chopped into small pieces. The tissue was incubated in the presence of 0.1% trypsin (200–300 U/mg) and 0.4% DNAseI for 5 min at 37°C, and the trypsin activity was inhibited by addition of 10% FBS in DMEM. After centrifugation at 4°C, the tissue was triturated in CMF-HBSS containing 1% DNAseI by using a P-1000 followed by a P-200 Pipetman pipette. The resulting cell suspension was centrifuged at 4°C, and the cell pellet was resuspended in CMF-HBSS. The cells were counted, and the viability was consistently found to be greater than 90% using trypan blue.

Cells were plated onto dishes, chamber slides, or glass coverslips in 24 well plates coated with 15 μg/ml polyornithine at a density of 45,000 cells/cm^2 in DMEM/10% FBS and incubated at 37°C under 5% CO_2. After 4 hr, the medium was replaced with a serum-free medum, DMEM/F12 (1:1), supplemented with 1 mM sodium pyruvate, 4 mM glutamine, 5 μg/ml insulin, 100 μg/ml transferrin, 100 μM putrescine, 20 nm progesterone, 30 nM sodium selenite, 3.7 g/liter sodium bicarbonate, and 100 U/ml penicillin-streptomycin (N2 medium; Bottenstein, 1985). In most of the experiments, bFGF was added at a concentration of 5 ng/ml. As a control, cells in which 100 μg/ml BSA instead of bFGF was added were grown in parallel.

Cells were grown in bFGF until confluent. For passaging, cells were incubated in the presence of 0.25% trypsin/2.5 mM EDTA for 15 min at 37°C and gently dispersed using a P-1000 Pipetman pipette. Trypsinization was inhibited by the addition of DMEM/10% FBS, and after centrifugation, the cells were replated in the presence of serum-containing medium. This medium, 4 hr later, was replaced by N2 medium containing 5 ng/ml bFGF. Cells growing in N2 medium in the presence of bFGF were tested for ability to differentiate after passage 2 or 3. bFGF was removed, and cells were washed and then incubated in N2 in the presence of 20 ng/ml BDNF, 20 ng/ml NT-3, 20 ng/ml NT-4/5, BSA, or bFGF for an additional 5 days.

Labeling Index Determination

Cells plated as described above were pulsed with BrdU at a final concentration of 10 μM for 18 hr immediately prior to fixation. Then, the cells were fixed with 4% paraformaldehyde/0.1 M borate ($Na_2B_4O_7$) buffer (pH 9.5) for 15 min, treated with 2 N HCl for 10 min, and neutralized in 0.1 M $Na_2B_4O_7$ for 10 min. Cells were incubated overnight with anti-BrdU monoclonal antibody (1:10). Then, the cells were incubated with horseradish peroxidase–conjugated goat anti-mouse secondary antibody (1:30) for 2 hr and developed using DAB–peroxidase reaction.

For thymidine incorporation, 2 μCi/ml [^{3}H]thymidine was added to the cultures for 18 hr. Then, the cells were washed with PBS, fixed with 10% tricloroacetic, and solubilized in 0.5 N NaOH. An aliquot of the extract was used to determine the counts incorporated.

Immunostaining of Cultured Cells

Immunocytochemistry was performed on E16 hippocampal cultures plated into 4 well plastic chamber slides or glass coverslips. On different days of culture, cells were fixed with 4% paraformaldehyde/0.1 M phosphate buffer (pH 7.4) for 10–15 min. After treatment with 0.1% Triton X-100/5% normal goat or horse serum/PBS, the cells were incubated for 2 hr to overnight with the primary antibodies against nestin (1:1000), MAP2 (1:500; supernatant of producing cells), calbindin-D_{28kDa} (monoclonal, 1:500; polyclonal, 1:1000), synapsin I (1:1000), or GFAP (1:50). Then, the cells were incubated with the corresponding biotinylated secondary antibody (1:200) followed by avidin–biotin–horseradish peroxidase complex (Vectastain ABC kit) and developed using DAB. For double immunocytochemistry, fluorescein- and rhodamine-conjugated secondary antibodies (1:100) at room temperature were used (1 hr). Coverslips and slides were mounted in Immu-mount or in DABCO/glycerol.

Morphological Analysis of Calbindin-Expressing Cells

The methods and equipment used for video microscopy were the same as described previously (Smith, 1994). Cultures were examined on a Zeiss Axiovert set up for differential interference contrast optics with a 40 × objective. Images were collected with a Newvicon video camera (Dage) processed to enhance contrast using a computer-based system (Image 1; Universal Imaging). Cell body area, length of longest process, and number of processes and branches of the cells were measured.

Growth Factors, Blocking Antibodies, and Drugs

bFGF was purchased from R & D Systems (Minneapolis, MN); BDNF and NT-3 were kindly provided by Dr. G. Yancopoulos (Regeneron Pharmaceuticals, Tarrytown, NY); NT-4/5 was purchased from Pepro-Tech (Rocky Hill, NJ). All growth factors were diluted in PBS containing 100 μg/ml BSA. The same solution was used as the BSA controls. Growth factors were added to the cultures and replaced every 2–3 days.

Blocking antibody against NT-3 was a gift from Amgen (Thousand Oaks, CA). Blocking antibody against bFGF was purchased from Upstate Biotechnology Incorporated (Lake Placid, NY). According to the supplier, anti-bFGF does not cross-react with acidic FGF. They were added to the cultures at 1:100 dilution and replaced every day.

Expression of the Results and Statistical Analysis

To determine the number of cells, a total of 10 fields equivalent to 500–2500 cells per sample were counted using 100 ×, 200 ×, or 400 × magnification under a phase or fluorescence microscope (Zeiss Axioplan). Results are expressed as percentage of cells or as the total number of cells expressing a specific marker in 10 fields. For each condition, 4–5 samples were done, and the data are the average ± SEM of two experiments. Morphological analysis was carried out on 30 random cells per condition. Statistical analysis were carried out using Student's t test.

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